In vitro reduction of methane production by 3-nitro-1-propionic acid is dose-dependent[1]

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ABSTRACT: Methanogenesis is a metabolic process that allows the rumen ecosystem the ability to maintain the low hydrogen partial pressures needed for proper digestive function. However, rumen methanogenesis is considered to be an inefficient process because it can result in the loss of 4% to 12% of the total energy consumed by the host. Recent studies have shown that some short-chain nitrocompounds such as nitroethane, 2-nitroethanol, 2-nitro-1-propanol, and 3-nitro-1-propionic acid (**3NPA**) are capable of inhibiting the production of methane during in vitro culture; nevertheless, optimal supplementation doses have yet to be determined. In the present study, in vitro cultures of freshly collected mixed populations of ruminal microbes were supplemented with the naturally occurring nitrocompound, 3NPA, to achieve 0, 3, 6, 9, or 12 mM. Analysis of fermentation products after 24 h of incubation revealed that methane ($\mathbf{CH_4}$) production was reduced in a dose-dependent manner by 29% to 96% ($P < 0.05$) compared with the amount produced by untreated controls (15.03 ± 0.88 μmol mL^{-1} incubated liquid). Main effects of the supplement were also observed, which resulted in a reduction ($P < 0.05$) on amounts of total gas and volatile fatty acids (VFA) produced, as well as in an increase of 0.07 to 0.30 μmol mL^{-1} on rates of 3NPA degradation. Changes in production of metabolites as CH_4, hydrogen ($\mathbf{H_2}$), VFA, and NH_3 indicated that the fermentation efficiency was not compromised dramatically by 3NPA treatment in moderate doses of 6 and 9 mM. Results further revealed that the metabolism of the 3NPA by microbial populations is also dose-dependent. The microbes were able to metabolize more than 75% of the added nitrocompound, with the greatest degradation rates in cultures treated with 9-mM 3NPA. Finally, from a practical standpoint, and considering the magnitude of CH_4 reduction, effect on VFA, and percentage of metabolized supplement, the most efficacious dose for 3NPA administration may be between 3 and 9 mM.

Key words: greenhouse gases, methane, nitrocompounds, rumen fermentation

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J. Anim. Sci. 2019.97:1317–1324
doi: 10.1093/jas/skz012

INTRODUCTION

Methanogenesis is a biochemical pathway used by certain microorganisms to produce methane ($\mathbf{CH_4}$; Wilkinson, 2012). Methanogenic bacteria use byproducts released by the fermentation of complex feedstuffs to reduce carbon

[1]We wish to thank the Consejo Nacional de Ciencia y Tecnología for the MS scholarship awarded to P.A.O-G. through the 385352 grant.

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Received September 7, 2018.
Accepted January 8, 2019.

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dioxide (**CO_2**) to CH_4 (Zehnder and Brock, 1979). In nature, this normal process helps anaerobic ecosystems maintain low partial pressures of hydrogen (**H_2**), thereby allowing a mechanism to dispose of the excess of reducing equivalents that otherwise would accumulate during anaerobic fermentation (Van Nevel and Demeyer, 1996). However, ruminal methanogenesis is also considered a digestive inefficiency because it can lead to a loss of 4% to 12% of the total energy consumed by the host (Johnson and Johnson, 1995). Moreover, CH_4 emitted by livestock to the atmosphere represents a serious environmental problem as it is the second most abundant greenhouse gas, after CO_2, generated from anthropogenic sources and is 21 times more potent than CO_2 in trapping heat in the atmosphere (IPCC, 2007; Harper et al., 2007).

A number of strategies have been proposed to mitigate economic and environmental costs associated with rumen CH_4 emissions. Strategies that increase animal production efficiency, such as the feeding of higher quality diets (McAllister et al., 1996), optimizing grazing management (McCaughey et al., 1997), or the utilization of genetically superior animals (Pinares-Patiño et al., 2003; Hegarty et al., 2007), can decrease CH_4 emissions by producing more meat or milk per equivalent or lesser amounts of CH_4 emitted. A number of these management strategies can reduce CH_4 production by as much as 30% compared with unmitigated practices. Additionally, even greater decreases in CH_4 emissions may be achieved by accompanying these management strategies with the addition of ruminal fermentation modifiers such as bypass fat (Beauchemin et al., 2006), tannins (Carulla et al., 2005), saponins (Hess et al., 2003), essential oils (Evans and Martin, 2000; Patra and Yu, 2012), and ionophores (Callaway et al., 2003). However, addition of these rumen fermentation modifiers would also add costs to animal production. In addition, many of these rumen modifiers have the further disadvantage of not being solely selective against methanogenic microorganisms but rather can exert their effects against a number of other ruminal microbes important for optimal rumen function (Patra and Yu, 2012). Additionally, supplementation with ionophores or other antibiotics is now prohibited in the European Union (Gustafson and Bowen, 1997). Clearly, alternatives are needed to mitigate rumen CH_4 emissions; yet, these must consider avoidance of increases in the production and management costs and ensure food product safety. Several studies have shown that under in vitro conditions some short-chain nitrocompounds such as nitroethane (**NE**), 2-nitroethanol (**2NEOH**), 2-nitro-1-propanol (**2NPOH**), and 3-nitro-1-propionic acid (**3NPA**) can inhibit the production of CH_4 (Anderson et al., 2003, 2008; Gutierrez-Banuelos et al., 2008; Božic et al., 2009). Moreover, NE and 2NPOH are effective under in vivo conditions (Anderson et al., 2006; Brown et al., 2011). However, NE, 2NEOH, and 2NPOH are synthetic compounds and none of them represent an alternative to be implemented on a commercial scale in the short term due to limited research about food safety issues. Also, the end products of their reduction (aminoethane, ethanolamine, and aminopropanol, respectively) have little or non-nutritional value for ruminants. In contrast, 3NPA is a natural compound produced in some fungi and plants and has been demonstrated to decrease CH_4 production up to 90% under in vitro conditions (Anderson et al., 2008). Additionally, its metabolism within the rumen leads to the production of β-alanine which is a nonessential amino acid that can be used by the animal. However, the level (12 mM) of supplementation evaluated previously may exceed the amount needed to be safe, efficacious, and economical for use under in vivo conditions (Anderson et al., 2008). Therefore, the objective of this study was to evaluate the effect of supplementation of different levels of 3NPA on the production of CH_4 and rumen fermentation parameters under in vitro conditions.

MATERIALS AND METHODS

Animal Management and Ruminal Fluid Sampling

Animals were maintained and cared for according to procedures approved by the University of Chihuahua animal care and use guidelines and the Mexican Government guidelines for the use of research animals (NOM-062-ZOO-1999). Ruminal fluid used as the source of rumen microbes was collected from 2 crossbreed Angus × Hereford heifers fed a basal diet based on oat hay and corn silage. Samples of rumen contents were obtained in 500 mL of ruminal fluid collected before the first feeding (0800 h), through a ruminal cannula surgically fitted to the heifers. The ruminal fluid was filtered through 4 layers of cheesecloth into a collection vessel kept in anaerobic conditions using CO_2 until the use (Paynter and Hungate, 1968). Mixed ruminal microbes were incubated in vitro for 24 h at 39 °C and shaken at 150 rpm using a reciprocal-shaking incubator (MaxQ 8000, Thermo Scientific, Waltham, MA).

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Experimental Design

The effect of the supplementation with 3, 6, 9, or 12 mM of 3NPA (Sigma-Aldrich Chemicals, Inc., St. Louis, MO) was evaluated. Each experimental unit was designated as an 18 × 150 mm crimp-top culture tube (by triplicate) containing 9 mL of basal medium which was composed by 40% clarified rumen fluid, and (mg•100 mL^{-1}) K_2HPO_4, 22.5; KH_2PO_4, 22.5; $(NH_4)_2SO_4$, 45; NaCl, 45; $MgSO_4$•$7H_2O$, 4.5; $CaCl_2$, 2.25; resazurin, 0.1; cysteine-HCl, 50; and Na_2CO_3, 400, plus 200 mg of ground alfalfa hay as substrate according to Gutiérrez-Bañuelos et al. (2008) and modified to contain a 100% CO_2 atmosphere. Each culture tube was inoculated by adding 1 mL of the freshly collected ruminal fluid, immediately sealed with rubber stoppers, and crimped closed with aluminum rings. Treatments were supplemented with 0.3 mL of stock solution of each 3NPA concentration or distilled water for the negative controls, which were administered via injection through rubber stoppers. The tubes were incubated at 39 °C and shaken at 150 rpm. After 24 h of incubation, total gas production and composition (CH_4 and H_2) were determined via analysis of the gaseous headspace. Fluid aliquots from each tube were also collected at 0, 3, 6, 12, and 24 h postincubation to measure 3NPA concentration. Total volatile fatty acids (VFA) production was determined subtracting the initial amount (0 h) from the amount after 24-h incubation.

Quantification of 3NPA and Qualitative β-Alanine Production

The concentration of 3NPA was analyzed colorimetrically according to Majak et al. (1982) using a spectrophotometer (Multiskan Go, Thermo Scientific, Waltham, MA). Fluid aliquots were centrifuged at 10,000 × *g* for 10 min, and supernatant was recovered. A volume of 50 mL from supernatants and standard solutions (3NPA standard) was mixed with 100 mL of NaOH 0.65 M. Subsequently, 100 mL of diazotized p-nitroaniline was added to the previous mixture. Finally, 2.5 mL of distilled water was added to the mixture and the absorbance was measured at 405 nm.

Quantitative β-alanine production analysis was carried out via thin layer chromatography, placing the final incubation products of each tube in 20 × 20 cm DC-Fertigplatten Cellulose F-coated plates (E. Merck, AG, Darmstadt, Germany). A mixture of butanol:acetic acid:water (4:2:1.2) was used as drain solvent and β-alanine 12 mM standards were run for comparison. Amines produced were further visualized by spraying plates with 0.2% ninhydrin (in ethanol) and letting it dry by heating at 110 °C for 10 min.

Analysis of Fermentation Products

Total gas production was determined by measuring volume displacement in a 30-cc glass syringe in each experimental tube after 24 h of incubation. Gas composition was determined by gas chromatography according to Allison et al. (1992), using a GOW-MAC chromatograph Series 580 (Gow-Mac Instrument Co., Bethlehem, PA). Volatile fatty acid production was quantified by gas chromatography according to Galyean (1989). Briefly the samples (5 mL) were mixed with 1 mL of meta-phosphoric acid [25% (wt/vol) meta-phosphoric acid] solution containing 2 g L^{-1} of 2-ethyl butyric acid and incubated in cold (ice bath) for 30 min. After the incubation period, the samples were centrifuged at 10,000 × *g* for 10 min at 4 °C. The quantification was done in a Clarus 400 Perkin Elmer Chromatograph (PerkinElmer, Waltham, MA) using a capillary column of stainless steel Poropak-Q of 30 m length; helium was used as a carrier gas (20 mL min^{-1}). Ruminal ammonia-N was determined using the phenol-hypochlorite procedure described by Chaney and Marbach (1962). Briefly, 50 mL from each sample or standard was mixed with 3 mL of phenol and 3 mL of hypochlorite reagents. The absorbance at 630 nm was measured using a spectrophotometer (Multiskan Go, Thermo Scientific, Waltham, MA).

Statistical Analysis

For statistical analysis, a standard one-way classification model for a completely randomized design was adjusted and orthogonal polynomial contrasts were used to examine linear and quadratic effects of treatment (3NPA level) on VFA, CH_4, and NH_3 and degradation rate using PROC GLM of SAS (Statistical Package, SAS Institute Inc., Cary, NC). Since H_2 accumulation was not detected for control and 3 mM of 3NPA treatments, data for the rest of treatments were analyzed by general analysis of variance and Tukey separation of means were performed using the software SAS (Statistical Package, SAS Institute Inc., Cary, NC). For 3NPA concentration through time, main fixed effects of 3NPA treatment, sampling time, and their interaction, as well as random effect of culture tube, were included in the model. PROC MIXED of SAS (Statistical

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Package, SAS Institute Inc., Cary, NC) was used for the statistical analysis. Since the interaction of treatment by sampling time was significant, a quadratic regression was fitted for 3NPA concentration through time for each treatment. For the 3-mM treatment, the regression was only adjusted until the 12th hour, when the total 3NPA was already metabolized.

RESULTS AND DISCUSSION

Degradation of 3NPA

The interaction of treatment by sampling time was significant ($P < 0.05$) following a quadratic tendency. A decrease in 3NPA concentration was observed as early as 3 h postincubation in all treated cultures, thus confirming the presence of competent 3NPA-degrading microbes within the ruminal populations (Figure 1). A linear increase ($P < 0.05$) in rates of 3NPA degradation was observed in response to augmenting 3NPA concentration levels. The rate of degradation (Table 1) significantly increased ($P < 0.01$) upon continued incubation suggesting an induction of 3NPA-degrading activity or enrichment in numbers of 3NPA-degrading microbes during the incubation. The most rapid degradation (0.30 μmol h^{-1}) occurred in cultures treated with 9-mM 3NPA, which suggests that this concentration was sufficient to saturate 3NPA-degrading activity within the populations in the present experiment. These findings are consistent with those reported in earlier studies (Majak, 1992; Anderson et al., 1993; Majak et al., 1998), which concluded that nitrocompound degradation can be improved via exposure to nonlethal concentrations of 3NPA or 3NPA-containing forages. This behavior could be explained by the increase in the concentration of *Denitrobacterium detoxificans*, which is recognized as the principal nitrocompound-reducing ruminal microorganism (Anderson et al., 2000). *Denitrobacterium detoxificans* is an obligate anaerobic bacterium that can oxidize reducing substrates such as H_2, formate, or lactate to reduce 3NPA to β-alanine, or a variety of other oxidized nitrocompounds to their respective amines, and thus has the potential to outcompete methanogens for available reductants (Anderson et al., 1993; Anderson and Rasmussen, 1998).

Gas Production

Total gas production measured after 24 h of incubation was linearly reduced ($P < 0.01$) when 3NPA concentrations were increased with treatments up to 12 mM (Figure 2) reaching a reduction of 1.9 mL^{-1} of gas per each 1 mM increased in the ruminal medium. On the other hand, a quadratic effect ($P < 0.01$) of 3NPA treatment level was observed on amounts of CH_4 produced by the mixed ruminal cultures. The cultures treated with 3-mM 3NPA produced 29% less CH_4 compared with that produced by untreated controls. However, the CH_4 produced was decreased much more dramatically (80% to 99% compared with controls) in cultures treated with 6-, 9-, or 12-mM 3NPA (Figure 3). Anderson et al. (2008) reported similarly dramatic decreases in CH_4 production when ruminal microbes were treated with 12-mM 3NPA, with production being decreased to near their detection limit (0.10 μmol mL^{-1}). In the present study, however, the most dramatic magnitude of inhibition of methanogenesis was observed when the supplemental 3NPA level was increased from 3- to 6-mM 3NPA, CH_4 production was decreased by 7.63 μmol mL^{-1} (Figure 3), compared with 4.42 μmol mL^{-1} when supplemental 3NPA level was increased from 0 to 3 mM. The decrease in CH_4 production was only marginally better with the 9- and 12-mM 3NPA treatments, achieving additional

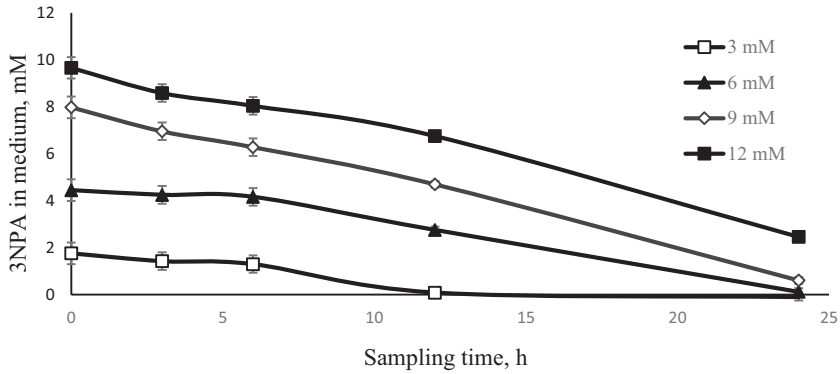

Figure 1. Quadratic regression of the 3NPA concentration (mM) on mixed populations of ruminal microbes through the sampling time with different treatment levels of 3-nitro-1-propionic acid (3NPA). Dots in figure are means ± standard deviation of observed values.

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Table 1. Degradation of 3-nitro-1-propionic acid (3NPA) under increasing supplementation level and their effect on fermentation products from mixed populations of ruminal microbes during 24 h of incubation

	3NPA in cultures, mM				Fluid measurements, μmol mL^{-1}					
Treatment	Initial concentration	Final concentration	% Metabolized	Degradation rate[1]	Total VFA	Acetate	Propionate	Butyrate	Ac:Pr ratio[2]	Ammonia
Control	NA	NA	NA	NA	77.30	60.67	13.70	2.93	4.45	10.88
3 mM	1.79	0.0	100	−0.075	48.70	36.59	9.75	2.36	3.74	10.55
6 mM	4.48	0.12	97	−0.188	66.29	47.83	14.02	4.44	3.41	10.87
9 mM	8.01	0.60	92	−0.303	66.99	48.95	14.17	3.86	3.46	11.25
12 mM	9.70	2.46	75	−0.293	61.54	43.51	14.30	3.74	3.07	12.93
Linear, *P*	NA	NA	NA	<0.0001	0.21	0.025	0.04	0.005	0.0017	0.08
Quadratic, *P*	NA	NA	NA	NS	0.03	0.02	0.19	0.003	0.26	0.2
SEM	NA	NA	NA	0.016	3.172	2.634	0.769	0.282	0.227	0.802

[1]mM of 3NPA degraded per hour.

[2]Acetate:propionate ratio.

NA = Not applicable; NS = Not significant.

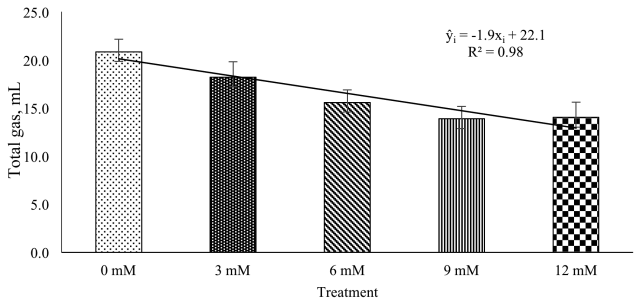

Figure 2. Total gas produced by mixed populations of ruminal microbes after 24-h incubation with different treatment levels of 3-nitro-1-propionic acid (3NPA). Values are means ± standard deviation. Polynomial orthogonal contrast analysis indicates a linear effect ($P < 0.01$) of total gas reduction.

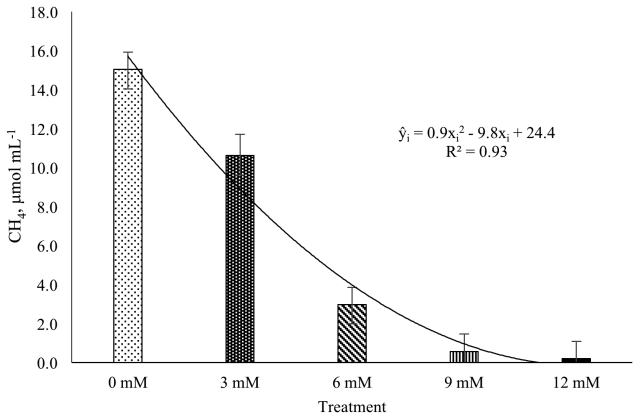

Figure 3. Methane produced by mixed populations of ruminal microbes after 24-h incubation with different treatment levels of 3-nitro-1-propionic acid (3NPA). Analysis with polynomial orthogonal contrasts indicates a quadratic effect ($P < 0.01$).

decreases of <2.78 μmol mL^{-1}. This suggests that from a practical standpoint, the most efficacious dose for 3NPA administration could be between 3 and 9 mM as this may achieve a balance between potency and toxicity. Also at that supplemental level (3 to 9 mM), at least 92% of the nitrocompound is metabolized (Table 1). Additionally, results from this and the earlier study by Anderson et al. (2008) suggest that the antimethanogenic capacity of the 3NPA, a natural product, is equivalent to that of the xenobiotic compounds NE and 2NEOH and better than 2NPOH.

The accumulation of H_2 after 24 h of incubation in the treatment supplemented with 3-mM 3NPA and the untreated controls was not detected. However, accumulations of H_2 in the cultures treated with 6-, 9-, and 12-mM 3NPA were greater ($P < 0.05$) compared with the control (0.27, 0.24, and 0.26 μmol mL^{-1}, respectively). Previous studies showed that 18% of the H_2 used in the production of CH_4 could come from the oxidation of formate catalyzed by formate dehydrogenase or formate hydrogen lyase activity, the latter being more active at lower concentrations of formate (Asanuma et al., 1999; Anderson et al., 2008; Gutiérrez Bañuelos et al., 2008). Thus, electrons released as H_2 or shuttled via other reduced carriers would be used by the methanogenic bacteria for the reduction of CO_2 to CH_4 (Hungate et al., 1970; Asanuma et al., 1999). Accordingly, the accumulation of H_2 in the present study was expected to follow an inverse tendency to the accumulation of CH_4; however, the results showed only a limited accumulation of H_2 (<0.278 μmol mL^{-1}), yet still higher than the 1-μM (0.1 kPa) concentration typically observed within the rumen. Feedback inhibition of H_2-producing hydrogenase activity is reported to occur at H_2 concentrations of approximately 1 kPa (Van Nevel and Demeyer, 1996), which would be equivalent to 0.62 μmol H_2 mL^{-1} under the conditions of the present experiment; this mechanism of inhibition may be improbable. It has been reported (Angermeier

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and Simon, 1983) that 2NEOH can inhibit the hydrogenase-catalyzed production of H_2 by inactivation of electron transfer capability of ferredoxin by *Clostridium pasteurianium*. More recently, a number of short-chain nitrocompounds have been reported to inhibit H_2 and formate oxidation but the mechanism has yet to be elucidated (Anderson et al., 2008). Thus, the limited accumulation of H_2 encountered could also be due to an inhibition of formate dehydrogenase caused by 3NPA or possibly via redirection of electrons released from formate oxidation to the reduction of 3NPA to β-alanine or to other alternative electron sinks. The metabolism of 3NPA to β-alanine was confirmed by thin layer chromatography, which showed the accumulation of β-alanine in the samples collected from treated cultures after 24-h incubation (not shown). Based on earlier stoichiometry reported by Angermeier and Simon (1983), for the reduction of nitroethanol to aminoethanol, the reduction of 3NPA to β-alanine would be expected to consume 3-μmol H_2 equivalents per mol of 3NPA which would not explain a considerable amount of reductant spared from the reduction of CO_2 to CH_4. The presence of other endogenous electron accepting compounds, such as oxidized sulfur or thio-containing proteins, nitrate, or unsaturated fatty acids, may also have consumed some of the electrons at the expense of CH_4 production, usually via oxidation of formate, lactate, or H_2 (Sparling and Daniels, 1990; Morgavi et al., 2010).

Concentration of VFA and Ammonia

Total VFA production decreased in all the treatments after 24-h incubation (Table 1) following a quadratic manner ($P < 0.01$). Cultures treated with 3- and 12-mM 3NPA showed a decrease of 37% and 20% in total VFA production, respectively, when compared with untreated controls. A similar tendency was observed for acetate. The accumulations of propionate were increased linearly by the treatments ($P < 0.05$). This finding is consistent with earlier observations by Lila et al. (2004) of effects of CH_4 inhibitor β-cyclodextrin diallyl maleate on ruminal VFA production. It is what typically happens with most other rumen CH_4 inhibitors previously investigated, largely because in the absence of a suitable electron acceptor, the electrons spared from reducing CO_2 can be directed to propionate synthesis (Lila et al., 2004). It has been reported that electron flow to propionate synthesis is promoted at partial pressures of $H_2 \geq 2.4$ kPa or the equivalent of 0.267 to 1.48 μmol H_2 mL^{-1} (Schulman and Valentino, 1975). A quadratic tendency was observed for butyrate ($P < 0.05$) reaching the maximal production when the cultures were treated with 6-mM 3NPA (Table 1).

These results provide evidence of the inhibition of the ruminal fermentation caused by 3NPA. Similar results have been observed by Beauchemin and McGinn (2006) and Ungerfeld et al. (2007) when several CH_4 inhibitors were used; they also observed a reduction on dry mater digestibility as a consequence of the supplementation of antimethanogenic compounds, leading to a decrease in acetate production. However, the effect of the antimethanogenic compounds on the synthesis of VFA depends on several factors, such as the addition of formate and H_2 (Anderson et al., 2006, 2008). The ratio of acetate to propionate (Ac:Pr) decreased linearly ($P < 0.05$) as the concentration of 3NPA increased. This effect could be due to the increase of the other VFA on the expense of acetate (Table 1). It is known that supplementation with some CH_4 inhibitors such as 9,10-anthraquinone (Garcia-Lopez et al., 1996; Kung et al., 2003) and diallyl malate (Lila et al., 2004) leads to an increase in propionate production.

Accumulation of ammonia did not differ between treatments ($P > 0.05$) at the end of the incubation period. However, the mean values tended to increase linearly ($P = 0.08$) along with the concentration of 3NPA (Table 1). These results differ from those reported previously when 2NPOH, NE, and 2NEOH were used as supplement where ammonia accumulations were higher in the nitro-treated cultures than in control (Anderson et al., 2003).

CONCLUSIONS

Our results showed that the supplementation of 3NPA dramatically reduces CH_4 production under in vitro conditions, and their effect is dose-dependent. However, the effect is negatively extended to VFA production which could compromise the animal productivity. The mechanism of action of this CH_4 inhibitor remains unknown, but it could be due, at least partially, to a redirection of reductants needed for the reduction of CO_2 to CH_4 to the reduction of the nitrocompound to β-alanine as was observed in this experiment. At present, a major limitation for the use of 3NPA as feed supplement is the high cost of its application. Therefore, finding alternative, lower cost sources for the nitrocompound is a major challenge for researchers. In addition, more thorough knowledge of the effects of nitrocompound supplementation on the rumen

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microbial population is needed to better understand their effect on rumen fermentation. Finally, more research is needed to determine the effect on animal productivity and to learn the optimal and safe dose for use, since the complete metabolism of the nitrocompound will guarantee animal food product free of this compound and a better economic performance of the food product.

Conflict of interest statement. None declared.

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